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Value of *MLH1* and *MSH2* Mutations in the Appearance of Muir–Torre Syndrome Phenotype in HNPCC Patients Presenting Sebaceous Gland Tumors or Keratoacanthomas

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Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal-dominant disorder characterized by predisposition to colorectal cancer and extracolonic malignancies, frequent multiple primary tumors in the same patient, and early age of cancer onset. A main clinical variant of Lynch syndrome, Muir–Torre syndrome (MTS) is characterized by the association between one or more visceral malignancies, with at least one sebaceous skin tumor or keratoacanthoma. In our study, we have screened a cohort of 538 HNPCC patients, related to 57 HNPCC families, to detect sebaceous skin tumors and keratoacanthomas and the role of mismatch repair (MMR) genes, *MLH1*, *MSH2*, and *MSH6*, in their pathogenesis. Among the 57 HNPCC families, we have identified four MTS families and one suspected MTS family, in which sebaceous carcinoma was found in one HNPCC mutation carrier subject who did not show visceral malignancy. In four of these families, linked to two *MLH1* mutations and to two *MSH2* mutations, biomolecular characterization showed concordance among immunohistochemistry analysis and gene mutations. The evidences of our investigations show that *MLH1* and *MSH2* gene mutations have an equivalent etiopathological role both for Lynch syndrome and for MTS; hence, we propose a broadened clinical criteria for definition of Lynch syndrome that will include sebaceous adenoma, carcinoma, and keratoacanthoma.

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INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), also referred to as Lynch syndrome, is an autosomal-dominant-inherited disorder characterized by predisposition to colorectal cancer and extracolonic malignancies (especially endometrium, ovary, stomach, small bowel hepatobiliary tract, uroepithelian tract, and brain), frequent multiple primary tumors in the same patient, and early age of cancer onset (Lynch and Lynch, 2005). The minimal clinical requisites that should be satisfied for the diagnosis of Lynch syndrome are defined by the ''Amsterdam Criteria I'' (Vasen *et al.*, 1991) and ''Amsterdam Criteria II'' (Vasen *et al.*, 1999). Successively, biomolecular parameters such as microsatellite instability (MSI) have been included in Bethesda guidelines (Rodriguez-Bigas *et al.*, 1997) according to the evidences of MSI in HNPCC related to colorectal cancer. Recently, a revised Bethesda guidelines has modified the spectrum of tumors that could be subjected to MSI analysis, including also brain and some rare sebaceous skin tumors typically of HNPCC variants (Umar *et al.*, 2004). In fact, besides the Turcot's syndrome in which central nervous system malignant tumors are reported (Hamilton *et al.*, 1995), the other main clinical variant of Lynch syndrome is the Muir–Torre syndrome (MTS), which is characterized by the combination of sebaceous gland tumors of the skin and internal malignancies (Lynch *et al.*, 1981). The MTS diagnosis is based on the coexistence of at least one sebaceous skin tumor (sebaceous adenomas and carcinomas) or keratoacanthoma and one or more visceral malignancies (Schwartze and Torre, 1995). The most common internal malignancies is colorectal cancer, accounting for 50% of all primary cancer in MTS; another common site is the genitourinary tract but also hematologic and breast malignancies are reported. The recognition of rare sebaceous skin neoplasms is of crucial

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Abbreviations: HNPCC, hereditary non-polyposis colorectal cancer; IHC, immunohistochemistry; MMR, mismatch repair; MSI, microsatellite instability; MTS, Muir–Torre syndrome

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clinical relevance because it predicts the associated risk for internal malignancies, thus giving rise to suspicion of a hereditary mismatch repair (MMR) defect (Mangold *et al.*, 2004; Ponti *et al.*, 2005a, b).

Lynch syndrome and MTS variants share common biomolecular pathogenesis linked to MMR genes mutations (*MLH1*, *MSH2*, *MSH6*, and *PMS2*) (Wheeler *et al.*, 2000; Calvert and Frucht, 2002). As a consequence of their inactivation, cells show a generalized genome instability, which is particularly evident at microsatellite loci (MSI) (Peltomaki *et al.*, 1993; Thibodeau *et al.*, 1993). In particular, in *MLH1* and *MSH2* genes, the major proportion (80%) of pathogenetic mutation in HNPCC families has been detected (Liu *et al.*, 1996; Papadopoulos and Lindblom, 1997). Muir–Torre phenotype is not linked to particular biomolecular MMR genes alteration, although the large majority of constitutional mutations are found in *MSH2* and only three *MLH1* mutations are reported linked to MTS phenotype (Mangold *et al.*, 2004).

In this study, we have screened an HNPCC patients cohort for the occurrence of sebaceous gland skin tumors and/or keratoacanthomas for the recognitions and biomolecular characterization of MTS. The specific aim of this study is to clarify the role of *MLH1* and *MSH2* gene alterations in the appearance of MTS phenotype and the relations between this genodermatosis and Lynch syndrome.

RESULTS

We have searched for skin tumors typical of MTS disorder in a cohort of 538 HNPCC patients belonging to 57 families selected through Amsterdam criteria. Five HNPCC index patients (about 1%) with diagnosis of sebaceous gland tumors and/or keratoacanthomas were recognized. In one of them (case 1), the diagnosis of sebaceous carcinoma was not associated with visceral malignancies in the same patient, although the family had typical HNPCC features: hence, we have classified this HNPCC family as suspected MTS (Figure 1). In the other four families, the patients responded to criteria for MTS definition and had a diagnosis of sebaceous skin lesions concomitantly (1) or successively (4) to internal cancer development (Table 1).

The histologic diagnosis of skin lesions were: keratoacanthoma in one MTS family, and sebaceous adenomas, epithelioma, and carcinoma in the other three MTS and suspected MTS family. These skin lesions were, essentially, localized on the eyelid and on the back. The mean age at first diagnosis of skin tumors was 51.4 years. The mean age at diagnosis of colorectal cancer was 51 years. In addition to colorectal cancer, other visceral malignancies reported in MTS families were uterus, ovary, pancreas, and breast cancer (Table 1).

A total of four MMR genes constitutional mutations were reported in the five MTS patients as a result of tumor tissue analysis (Table 2). For all but one patient, we have examined sebaceous skin tumors and keratoacanthomas for microsatellite status and immunohistochemical expression of *MLH1*, *MSH2*, and *MSH6* proteins. The skin lesions belonging to four MTS probands showed MSI; moreover, in these cases, a concordance with immunohistochemical analysis was found (Figures 2 and 3). All visceral tumors in the MTS group have been studied; in all cases, MSI and lack of expression of at

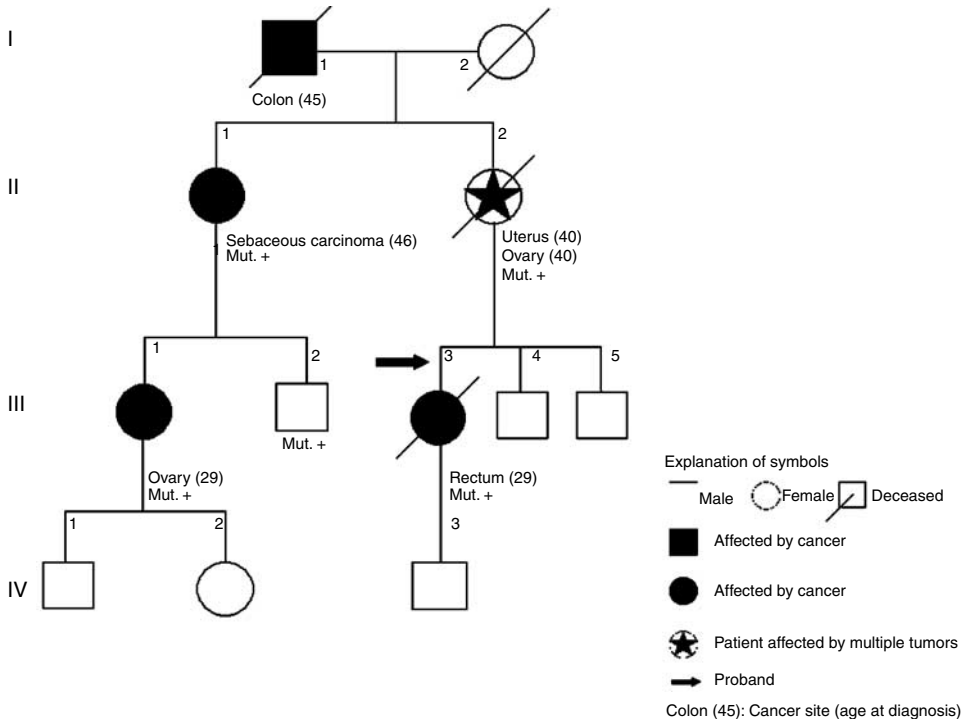

Figure 1. Genealogic three of suspected MTS linked to MSH2 mutations (del TT at 880 exo 5). A II-1 patient shows sebaceous carcinoma without visceral malignancy.

Table 1. Clinical features of MTS patients identified among HNPCC families

Case	Sex	Skin lesion histology	Age at first skin lesion	Site	Visceral tumor in the proband (age)	Tumors in the family (age)
1	F	Sebaceous carcinoma	46	Back	—	Uterus 40 (sister) Ovary 40 (sister) Ovary 34 (daughter) Rectum 29 (nephew)
2	M	Sebaceous epithelioma	49	Back	Colon 49	Rectum 59 (father) Colon 38 (mother)
3	M	Sebaceous carcinoma	54	Eyelid	Colon 48	Colon 45 (brother) Colon 38 (brother) Uterus 40 (sister) Bone 16 (nephew)
4	F	Sebaceous adenoma	46	Eyelid	Breast 38 Uterus 40 Colon 45	Colon 33 (mother) Colon 39 (sister) Colon 32 (brother) Stomach 42 (uncle) Colon 37 (uncle) Uterus 50; colon 48 (ant) Uterus 33 (grandmother)
5	F	Keratoacanthoma	63	Nose	Colon 62	Pancreas 60 (brother) Pancreas 50 (brothers) Breast (aunt) Colonic adenomas (daughter)

F, female; HNPCC, hereditary non-polyposis colorectal cancer; M, male; MTS, Muir–Torre syndrome.

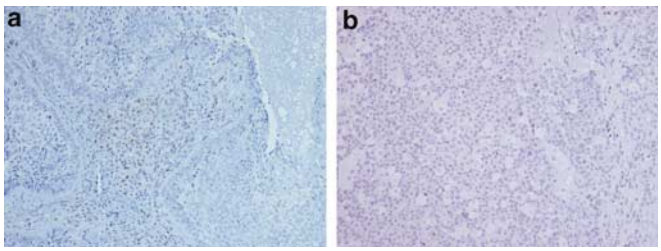

Figure 2. Immunohistochemical analysis of MMR proteins. (**a**) Lack of expression of MLH1 protein in sebaceous carcinoma. (**b**) Lack of expression of MSH2 protein in sebaceous carcinoma.

Figure 3. Clinical aspect of a keratoacanthoma in the MTS patient. Bar = 10 mm; lesion size: 24 × 18 mm.

least one of the MMR proteins was evident in the same patient. In three MTS patients, the evidences of immunohistochemistry (IHC) suggest the localization of a mutation in *MSH2/MSH6* gene (cases 1, 2, and 3) and in the other two in *MLH1* gene (cases 4 and 5).

The two *MSH2* mutations were del TT at 880 exo 5 (case 1) and a large deletion at exo 1 (case 2). As regards case 3, no other affected family members were available for segregation analysis; IHC showed lack of MSH2 and MSH6 protein expression and the tumor tissue was reported to be H-MSI (Table 2). One of the two *MLH1* mutations (case 4) was characterized as founder mutations in four HNPCC families distributed in the provinces of Modena and Reggio Emilia, but in the patient belonging to the other three related families with the same mutations, the appearance of sebaceous lesions or keratoacanthomas was not reported. The mutation consisted in the insertion of a T between nucleotides 2269 and 2270 (2269–2270 ins T), causing the synthesis of a longer (by 33 amino acids) but unstable polypeptide (Caluseriu *et al.*, 2004). The other *MLH1* mutation (case 5) was c.1520–1521 ins T, causing a frameshift and appearance of premature stop codons (Caluseriu *et al.*, 2001).

Table 2. Biomolecular features of MTS patients identified among HNPCC families

Case	Sex	MSI	IHC	Mutation analysis
1	F	H-MSI	Lack of expression of MSH2/MSH6	del TT at 880 exo 5 of hMSH2
2	M	H-MSI	Lack of expression of MSH2/MSH6	Large deletion exo 1 of hMSH2
3	M	H-MSI	Lack of expression of MSH2/MSH6	Not tested (proband deceased)
4	F	H-MSI	Lack of expression of MLH1	ins T 2269–2270 of hMLH1
5	F	H-MSI	Lack of expression of MLH1	c.1520–1521 ins T of hMLH1

F, female; HNPCC, hereditary non-polyposis colorectal cancer; IHC, immunohistochemistry; M, male; MSI, microsatellite instability; MTS, Muir–Torre syndrome.

DISCUSSION

The main evidence of our study was at first, a higher incidence of rare sebaceous skin tumors and keratoacanthomas in the HNPCC subjects' cohort with respect to the general population and consequently the improvement of the current clinical definition of this genetic disorder. The MTS cases identified in our case histories of HNPCC families show that the frequency of this phenotype is clearly underestimated probably owing to the scarce importance assigned to research and registering of sebaceous gland tumors and/or keratoacanthomas in personal and familiar anamnestic survey.

A clinical definition of MTS may result rather difficult as it requires the coexistence of at least one sebaceous tumor or keratoacancthoma with one or more visceral malignancy (Schwartze and Torre, 1995); this clinical condition may appear as sporadic MTS case in which a tumor family history is not reported and as an allelic variant of Lynch syndrome, for they share the same pathogenesis and similar clinical features (Ponti and Ponz de Leon, 2005). However, we believe that by including sebaceous tumors or keratoacanthomas in the clinical tumor spectrum of Lynch syndrome, the supervision and investigation would be performed on very small families whose full phenotype expression is difficult to estimate and on visceral disease-free subjects, as in the suspected MTS case examined herein (case 1). This approach could be particularly useful for those cases in which a lack of synchronous or metachronous coexistence in sebaceous gland tumors and visceral malignancies in the same subject would stop further mutational investigations and consequent clinical surveillance.

The original Amsterdam criteria were created for the necessity of homogenizing the cohort of colorectal cancer families for multicenter clinical and biomolecular study (Vasen *et al.*, 1991). Their limitations and low efficacy in the clinical applications were supplied by the Amsterdam Criteria II (Vasen *et al.*, 1999) that accounts in the clinical tumors spectrum of HNPCC and other extracolonic tumors such as endometrium, small bowel, ureter, or renal pelvis. In the same way, the original Bethesda guidelines (Rodriguez-Bigas *et al.*, 1997), in which criteria for the selection of colorectal tumors to be tested for MSI were present, has been integrated by revised Bethesda guidelines including extracolonic HNPCC-associated tumors. Subsequently, sebaceous gland adenomas and keratoacanthomas in MTS have been included in the tumors to be tested for MSI (Umar *et al.*, 2004). Among these, we propose to include also rare sebaceous carcinomas – that account for one in every 2,000 skin malignancies (Janjua *et al.*, 1997) – as we believe that these rare lesions should be tested even when they appear in individuals without any visceral malignancies. Kruse *et al.* (Kruse *et al.*, 2003; Ponti *et al.*, 2005a, b) suggested that to every patient with a sebaceous tumor should be offered either molecular genetic diagnostic or, if necessary, a strict regular cancer surveillance program. These rare sebaceous lesions will have to be researched and registered especially in HNPCC contests where MTS may form a variant.

The MTS tumors spectrum is well defined for the peculiar skin tumor, but some contentions exist about visceral tumor spectrum. In fact, with the exception of colorectal and urogenital cancers (Davis and Cohen, 1995; Ponti and Ponz de Leon, 2005), the role of other visceral malignancies needs further investigations, such as for the breast cancer that seems to be frequently reported among MTS-associated neoplasms (Cohen *et al.*, 1995). Although breast cancer has been occasionally reported in the HNPCC pedigree (Risinger *et al.*, 1992), several investigations show that it may represent phenocopies unrelated to the presence of MMR gene mutations (Anbazhagan *et al.*, 1999; Aarnio *et al.*, 1999; Vasen *et al.*, 1999; Caluseriu *et al.*, 2001).

The geno-phenotype characterization in MTS and suspected MTS cases shows that *MLH1* and *MSH2* have an equivalent etiopathological role and that there is no correlation between MTS mutations and particular functional domains inside these two genes. Therefore, the ''subordinate'' role of *MLH1* in the MTS genesis has to be re-evaluated and its involvement should always be investigated, together with *MSH2*, first through IHC analysis, and second through direct sequencing or deletion mapping. On the other hand, it seems rather unclear how, under the same mutation and family history, a MTS phenotype appears only in some cases: for instance, the *MLH1* founder case shows that the same mutation associated to phenotype MTS in our index patient does not match with such phenotype in other carriers of the same mutation, either in the same family or in other HNPCC-correlated families. These evidences could be owing to lower penetrance of *MLH1* mutations with respect to *MSH2* mutations that are usually reported to convey a higher risk for extracolonic cancer than *MLH1* (Hampel *et al.*, 2005).

Hence, further studies are needed to investigate thoroughly the relationship between the cutaneous cancerogenesis and the molecular alterations owing to MMR genes and their genomic instability, in particular for the cases of ''founder mutations'' inherited. Such as for the Lynch syndrome (Peltomaki and Vasen, 2004), even in MTS the known founder mutations could be first tested by IHC analysis in all sebaceous lesions or multiple keratoacanthomas, registered in the specific area potentially linked to ''founder mutation effects''.

Although the current definition of MTS identifies sporadic cases accurately, it does not include all those relevant family characteristics in MTS allelic variant of HNPCC; therefore, an additional ''broadened'' clinical definition of Lynch syndrome, including also sebaceous skin tumors and keratoacanthomas in the neoplastic spectrum, should be provided.

The molecular pathogenesis of the syndrome is caused by both MSH2 and MLH1, and whenever the tumor tissue shows MSI, this gene shall be investigated together with MSH2. In this way, also when associated visceral malignancies are absent, the mutational analysis in first-degree relatives of gene carriers may be directed to the isolation of the sole cut phenotype, so that the mutational research in these subjects may anticipate all those prevention strategies applied to visceral associated risk.

MATERIALS AND METHODS

The study was conducted according to the Declaration of Helsinki Principles and institutional approval and written informed patient consent were obtained.

HNPCC families

Screening for sebaceous gland tumors and keratoacanthoma was performed in 538 HNPCC patients identified from the data of specialized registry that was instituted in 1984 in the District of Modena (Northen Italy) (Ponz de Leon *et al.*, 1999) and through references from other areas of Italy. Since then, a total of 57 kindred with clinical features of HNPCC have been collected, and in 25 of them, germline mutations in *MLH1*, *MSH2*, and *MSH6* are reported. All the HNPCC families fulfilled the diagnostic Amsterdam I or Amsterdam II criteria for HNPCC definition.

MSI analysis

Paraffin-embedded tumor tissue and corresponding normal mucosa were microdissected with sterile scalpels into polypropylene tubes. The tumor and normal tissue were deparaffined with xylene, washed with ethanol, and dried up. The digestion was performed at 50°C overnight. The samples were then heated at 80°C for 10 minutes to inactivate the proteinase K and centrifuged. After purification with NaCl-saturated solution and precipitation in absolute ethanol, the supernatant was used as template for PCR amplification. MSI status was determined by using five fluorescent-labelled microsatellite markers (BAT25, BAT26, D2S123, D5S346, and D17S250, the Bethesda Panel). PCR reactions were carried out in a 10 μl reaction volume containing 50–100 ng of genomic DNA, 0.15 pmol of dye-labelled forward and unlabelled reverse primers, 2 mM concentration of each deoxynucleotide triphosphate, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), and 0.6 U of *Taq* polymerase. PCR products were run on a CEQ 8000 Sequencer (Beckman Coulter, Milano, Italy), and analyzed using the Fragment Analysis System by Beckman Coulter. Lesions were scored as MSI+ when instability could be detected in at least two microsatellite loci. A family was considered MSI+ when 50% or more of the investigated patients showed MSI in the researched neoplasms.

IHC analysis

Immunohistochemical analysis of MSH6, MLH1, and MSH2 proteins were carried out on paraffin-embedded tumor samples. Immunoperoxidase staining, using diaminobenzidine as chromogen, was run with the NEX-ES Automatic Staining System (Ventana, Strasbourg, France). Mouse mAbs were anti-MSH6 (Transduction Labs, BD Biosciences, Milano, Italy) at 1:2,000 dilution, anti-MLH1 (Pharmingen, San Diego, CA) at 1:40 dilution, and anti-MSH2 (Pharmingen) at 1:40 dilution. Nuclei were counterstained with hematoxylin and adjacent normal tissue in each sample served as positive control.

Mutation analysis

The study of germline mutations in the main DNA MMR genes (*MLH1*, *MSH2*, and *MSH6*) were performed by direct genomic sequencing of DNA derived from blood leukocytes of the proband or other affected family members. Amplification products were generated with primers located in the flanking introns approximately 50 bp from the respective intron/exon borders to detect all possible splice junction mutations. The sequences were determined on a CEQ 8000 Sequencer (Beckman Coulter) and analyzed with the Sequence Analysis System by Beckman Coulter. At the beginning of the study, germline mutations of MMR genes were evaluated under previously reported conditions (Caluseriu *et al.*, 2004).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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